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Potentiation of Hypoosmotic Cellular Volume Regulation in the Quahog, *Mercenaria mercenaria*, by 5-hydroxytryptamine, FMRFamide, and Phorbol Esters*

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Abstract. Ventricles isolated from clams (*Mercenaria mercenaria*) that had been acclimated to 1000 mOsm seawater (SW) release amino acids when incubated in 500 mOsm SW. Taurine, glycine, and alanine account for nearly all of the released amino acids, and total about 37 μmol/g dry tissue weight during a 2-h incubation. The release of amino acids is increased to 69 μmol/g by the addition of 10^{-6} *M* 5-hydroxytryptamine (5HT) to the hypoosmotic SW, and to 83 μmol/g by the addition of 10^{-6} M FMRFamide to the medium. The potentiation of the release by 5HT is blocked by methysergide. The amino acid release is increased by two phorbol esters—phorbol 12,13-diacetate and phorbol 12-acetate, 13-myristate—to 97 and 83 μmol/g, respectively. Forskolin and other cyclic 3′,5′ adenosine monophosphate agonists have no effect on the release of amino acids in hypoosmotic SW. Phorbol esters, 5HT, and FMRFamide have no effect on the release of amino acids from ventricles incubated in 1000 mOsm SW. Ventricles, first isolated from clams acclimated to 1000 mOsm SW, and then transferred to 500 mOsm SW, increase in wet weight by 20–25%. The increase is maintained for 30 min, and the tissues return their original weight in the ensuing 30 min. The addition of 5HT, FMRFamide, or phorbol esters to the hypoosmotic SW decreases the time necessary for the tissues to return to pre-transfer weights. These results implicate protein kinase C in the responses of bivalve tissues to hypoosmotic media, and suggest that these responses may be modified by neuronal or neurohumoral control.

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Introduction

In osmoconforming marine bivalves, the restoration of cellular volume in response to changes in the ambient salinity is accomplished by the adjustment of cytoplasmic concentrations of ions and amino acids (Gilles, 1979; Pierce, 1982). The cells of these animals release amino acids when exposed to a hypoosmotic medium, thereby reducing the osmotic gradient between the medium and the cytoplasm (Pierce and Greenberg, 1972; Gainey, 1978; Amende and Pierce, 1980).

The extirpation of particular ganglia in bivalves has been reported to affect the water balance of the animals (Lubet and Pujol, 1963; Nagabushanam, 1964; Durchon, 1967). In both *Crassostrea virginica* and *Mytilus galloprovincialis*, putative neurosecretory cells lost their granular inclusions when the animals were exposed to hypoosmotic media (Lubet and Pujol, 1963; Nagabushanam, 1964). In the opisthobranch *Aplysia californica*, the electrical activity of cell R-15 in the abdominal ganglion is depressed by exposure of the whole animal to dilute seawater (Bablanian and Treistman, 1983). Ninety minutes after homogenates of R-15 were injected into an intact *A. californica*, the animal's wet weight increased by 5% (Kupfermann and Weiss, 1976). The gain in weight induced by the homogenate occurred even in a 5% hyperosmotic medium, in which the animals would be expected to lose water. Hyperpolarization of R-15 in intact animals causes large increases in the free amino acid content of the blood (Bablanian and Treistman,

1985). These observations, while far from conclusive, suggest that neurosecretory products might be involved in the regulation of osmotic balance in molluscs.

This paper describes the effects of known molluscan neurotransmitters and neurohormones on the hypoosmotic volume regulation of isolated ventricles of the clam *Mercenaria mercenaria*. In addition, the effects of cyclic 3′5′ adenosine monophosphate (c-AMP) and protein kinase C agonists on volume regulation have been investigated. The neuropeptide FMRFamide, 5-hydroxytryptamine, and phorbol esters, but not c-AMP agonists, potentiate hypoosmotic volume regulation.

Materials and Methods

Animals and media

Mercenaria mercenaria were collected from several locations in the Intracoastal Waterway in St. Johns County, Florida, or obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were maintained, unfed, in either running seawater (SW) (940–1000 mOsm), or in aerated aquaria containing natural SW (1000 mOsm). In all cases, the test media were compounded from vacuum-filtered (0.45 μm) natural SW. The 500 mOsm SW was made by dilution of filtered SW with deionized water.

Concentrated solutions of pharmaceuticals were prepared as follows: forskolin was dissolved in ethanol; phorbol, phorbol 12,13-diacetate, and phorbol 12-acetate,13-myristate were dissolved in dimethylformamide; all others were dissolved in deionized water.

The neural substances tested included the molluscan neuropeptides phenylalanyl-methionyl-arginyl-phenylalanylamide (FMRFamide), phenylalanyl-leucinyl-arginyl-phenylalanylamide (FLRFamide), para-glutamyl-aspartyl-phenylalanyl-leucinyl-arginyl-phenylalanyl-amide (pQDFLRFamide), small cardioactive peptide B (SCP_B), acetylcholine (Ach), and serotonin (5HT). The 5HT receptor blocker methysergide (UML 491) was also used. These drugs were added to either 1000 mOsm or 500 mOsm SW to obtain the desired concentration. Solvent carriers other than water were added to 1000 mOsm or 500 mOsm SW in the same amounts necessary to achieve the desired drug concentrations; such media were used to control for the effect of solvent.

Analysis of the tissue free amino acid pool

Ventricles were removed from animals that had been acclimated to 1000 mOsm SW, and lyophilized. The dried tissues were weighed and homogenized in a small volume of 80% ethanol (1 ml/5 mg dry tissue). The homogenates were centrifuged in a microcentrifuge and 20 μl of the supernatant fluid removed, evaporated to dryness in an oven (60°C), dissolved in 0.02 *N* HCl, and analyzed for amino acids with an amino acid analyzer (Hitachi 835, Na^+ citrate).

Measurement of release of amino acids

Ventricles were dissected from clams, placed in a large volume of 1000 mOsm filtered SW, and incubated for an hour. The ventricles were then placed in 2 ml of either 1000 mOsm or 500 mOsm SW. Preliminary studies of the time course of the release of amino acids from ventricles exposed to 500 mOsm SW showed that the levels of amino acid accumulated in the bath became maximal within 90–120 min. Consequently, a 2-h incubation time was chosen for subsequent experiments.

At the end of the incubation period, a 500-μl sample of the bath fluid was removed, acidified with 5 μl of 0.2 *N* HCl, and analyzed for amino acid content on an amino acid analyzer (Hitachi 835). A protein hydrolysate program was used; quantitation was provided by analyses of a standard mixture of amino acids (Pierce Chemical). Preliminary experiments showed that alanine, glycine, and taurine accounted for over 95% of the total amino acid release; the analysis program was therefore truncated to analyze only those amino acids eluting before cysteine. The amino acid levels are reported in this paper as the sum of alanine, glycine, and taurine, and are reported as μmol/g dry weight. In experiments involving the phorbol esters and the c-AMP agonists, the tissues were incubated in 1000 mOsm SW containing the desired concentration of the agent for 30 min, and were then transferred either to 1000 mOsm or 500 mOsm SW containing the same concentration of these agents.

Measurement of changes in wet weight

The ventricles were removed from clams and incubated in 1000 mOsm SW for an hour. The ventricles were blotted repeatedly on tissue paper until they left no bead of water when touched to a glass plate, and then weighed on an analytical balance (Sartorius 2463). The ventricles were then transferred to test tubes containing 2 ml of the test medium and weighed at intervals. This procedure produced repeatable weights for 3–4 weighings, but with further blotting and weighing, the tissue adhered to the blotting paper and a substantial amount (20–30% wet weight) was lost.

Statistical treatment of the data

Changes in wet weight were expressed as percentages of the original weight of the ventricle; these values were transformed by the arcsin transformation and means and standard deviations were computed. Differences in the mean changes in weight between control and treated

Table I

Amino acid content of ventricles from Mercenaria mercenaria *acclimated to 1000 mOsm seawater*

Amino acid	Content (μmol/g dry weight)
Taurine	415.8 ± 11.9
Aspartic acid	12.0 ± 4.1
Glutamic acid	32.1 ± 5.1
Glycine	27.9 ± 5.5
Alanine	30.9 ± 6.7
Arginine	8.2 ± 0.8
Others	8.0 ± 2.5
Total	534.4 ± 29.7

ventricles were assessed by Student's t test. Student's t test was also used to evaluate differences among the means of treatment groups in the dose-response experiments. The data for the release of amino acids by ventricles were analyzed by a one-way analysis of variance. Differences between treatment groups and the appropriate solvent control groups were assessed by *a priori* F tests.

Results

The amino acid content of ventricles from *Mercenaria mercenaria* is summarized in Table I. Taurine, glutamic acid, glycine, and alanine make up 95% of the total pool. Aspartate and arginine, as well as very small amounts (5 μmol/g or less) of proline, threonine, and serine also contribute to the total pool. Traces of the other neutral and basic amino acids were detected in some, but not all, tissues.

Taurine, glycine, and alanine account for about 80, 10, and 5%, respectively, of the total net loss of amino acids from isolated *Mercenaria* ventricles incubated in either 500 mOsm SW or 1000 mOsm SW. The effects of molluscan neurotransmitters and neurohormones on the release of the three amino acids from ventricles exposed to dilute medium are shown in Figure 1. Ventricles incubated in 500 mOsm SW release 37.1 μmol/g in 2 h. The addition of 10^{-6} *M* 5-hydroxytryptamine (5HT) to the dilute medium increases the net release of amino acids by 87%; the increase is significant ($F_{1,135} = 5.7$; $P < 0.05$). The molluscan neuropeptides FMRFamide, FLRFamide, and pQDFLRFamide, in concentrations of 10^{-6} *M* significantly increase the release of amino acids by 110% or more, but neither SCP_B nor acetylcholine have any effect (Fig. 1). The effect of 5HT on the amino acid release is blocked by the 5HT receptor blocker methysergide (UML); UML alone has no effect on the release of amino acids (Fig. 2).

Dose-response curves for the effects of 5HT and

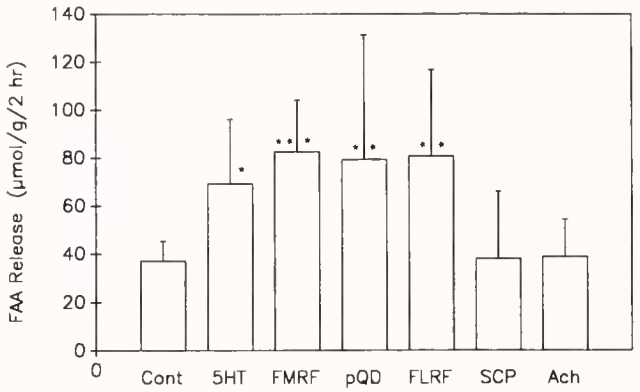

Figure 1. The effects of molluscan neural products on the release of amino acids from *Mercenaria* ventricles transferred from 1000 mOsm seawater to 500 mOsm seawater. Each bar represents the mean of 10 ventricles; error bars are 1 SD. Treatments: Cont = controls; 5HT = 5-hydroxytryptamine; FMRF = FMRFamide; FLRF = FLRFamide; pQD = pQDFLRFamide; SCP = SCP_B; Ach = acetylcholine. The concentration of each agent was 10^{-6} *M*. Each point is the mean ± SD, n = 9. Asterisks indicate treatments significantly different from controls: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

FMRFamide on the release of amino acids by ventricles in hypoosmotic seawater are shown in Figure 3. The difference in amino acids released between control ventricles and those exposed to 5HT and FMRFamide are significant at concentrations of 10^{-10} *M* (for 5HT, t = 5.78, $P < 0.001$; for FMRFamide, t = 2.57, $P < 0.05$) and above. Concentrations of 5-HT greater than 10^{-8} *M* elicit no further significant increase in the release of amino acids. The amino acid releases elicited by concentrations of FMRFamide from 10^{-10} to 10^{-6} *M* are not

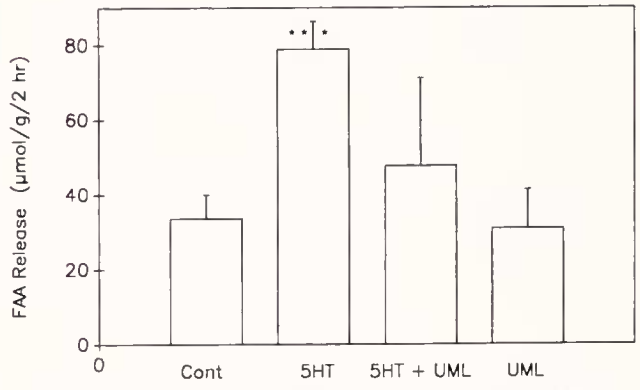

Figure 2. The effect of methysergide and 5-hydroxytryptamine on the release of amino acids from *Mercenaria* ventricles transferred from 1000 mOsm seawater to 500 mOsm seawater. Each bar represents the mean of five ventricles; error bars are 1 SD. Treatments: Cont = controls; 5HT = 5-hydroxytryptamine (10^{-6} *M*); 5HT + UML = 5-hydroxytryptamine (10^{-6} *M*) and methysergide (10^{-5} *M*); UML = methysergide (10^{-5} *M*). The asterisks indicate treatments significantly different from controls: *** = $P < 0.001$.

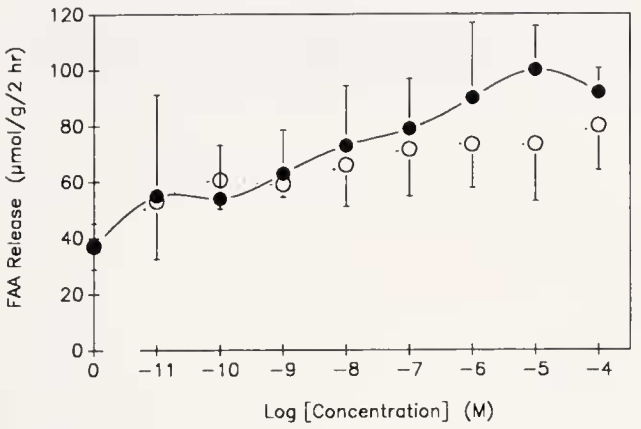

Figure 3. The effects of increasing concentrations of 5-hydroxytryptamine and FMRFamide on the release of amino acids from *Mercenaria* ventricles transferred from 1000 mOsm seawater to 500 mOsm SW. The point at zero on the abscissa represents the release from control tissues. Open circles = 5HT; solid circles = FMRFamide; Each point is a mean (n = 10 for controls, n = 4 for each treatment); the error bars indicate 1 SD.

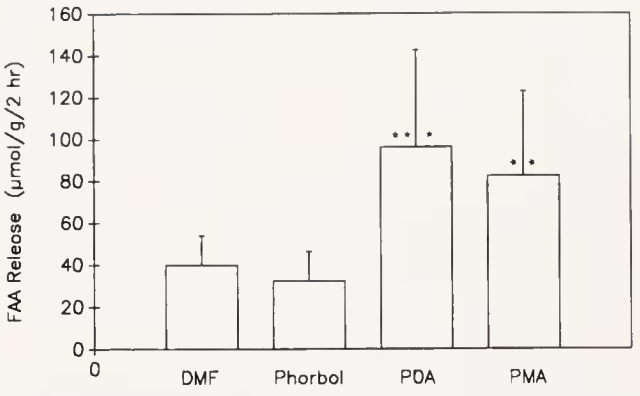

Figure 5. The effects of phorbol esters on the release of amino acids from *Mercenaria* ventricles transferred from 1000 mOsm seawater to 500 mOsm SW. Each bar represents the mean of 10 ventricles; error bars are 1 SD. Treatments: DMF = dimethylformamide (0.01%); Phorbol = 4-β-phorbol (10^{-7} *M*); PDA = phorbol 12,13-diacetate (10^{-7} *M*); PMA = phorbol 12-acetate,13-myristate (10^{-7} *M*). Asterisks indicate treatments that are significantly different from controls: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

significantly different, but the difference between tissues exposed to 10^{-10} and 10^{-5} *M* is significant (t = 3.22, $P < 0.01$).

The effects of several cyclic 3′,5′adenosine monophosphate (cAMP) agonists on the release of amino acids in hypoosmotic seawater are shown in Figure 4. None of these agents affect the amino acid release. The cyclic guanosine monophosphate agonist 8-bromo-cyclic GMP also has no effect on the release of amino acids from ventricles in 500 mOsm SW (data not shown). In contrast, two phorbol esters potentiate the amino acid release, while 4-β phorbol has no effect (Fig. 5). The effective esters, phorbol 12,13-diacetate and phorbol 12-acetate,13-myristate, increase the release of amino acids by 140 and 106%, respectively; the comparisons were made to ventricles incubated in hypoosmotic seawater containing dimethylformamide (1.4 m*M*).

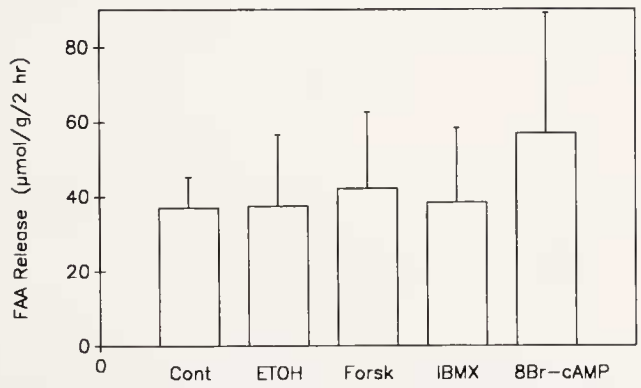

Figure 4. The effects of adenosine 3′-5′cyclic monophosphate agonists on the release of amino acids from *Mercenaria* ventricles transferred from 1000 mOsm seawater to 500 mOsm SW. Each bar represents the mean of 10 ventricles; error bars are 1 SD. Treatments: Cont = controls; ETOH = ethanol (0.1%); Forsk = forskolin (10^{-5} *M*); IBMX = 3-isobutyl-1-methylxanthine (10^{-3} *M*); 8Br-cAMP = 8-bromo-adenosine 3′-5′cyclic monophosphate (10^{-3} *M*).

The effects of 5HT, selected molluscan neuropeptides, and the phorbol esters on the release of amino acids from ventricles incubated in isosmotic seawater for 2 h are shown in Figure 6. The release of amino acids from ventricles in isosmotic SW is considerably lower than that from tissues exposed to hypoosmotic SW. None of the molluscan neural products affects the release of amino acids from tissues in isosmotic medium. The release of amino acids in the presence of phorbol esters and 4-β phorbol is not different from that of ventricles incubated in 1000 mOsm SW containing dimethylformamide (1.4 m*M*). The release of amino acids from ventricles incubated in dimethylformamide is significantly higher than that of control tissues incubated in 1000 mOsm SW.

The changes in wet weight experienced by ventricles incubated in hypoosmotic seawater containing 5HT, FMRFamide, and phorbol esters are shown in Figure 7. Ventricles transferred from 1000 mOsm SW to 500 mOsm SW gained about 20–30% in wet weight within 10 min; this gain is maintained for 30 min, and then gradually decreases to zero over the following 30 min. The addition of FMRFamide (10^{-6} *M*) to the bathing medium reduces the time required by the tissues to regulate volume (Fig. 7a). The changes in wet weight of tissues incubated in media containing FMRFamide are significantly lower than those of control tissues at both 30 (t = 10.1, $P < 0.001$) and 60 min (t = 5.69, $P < 0.001$) after transfer.

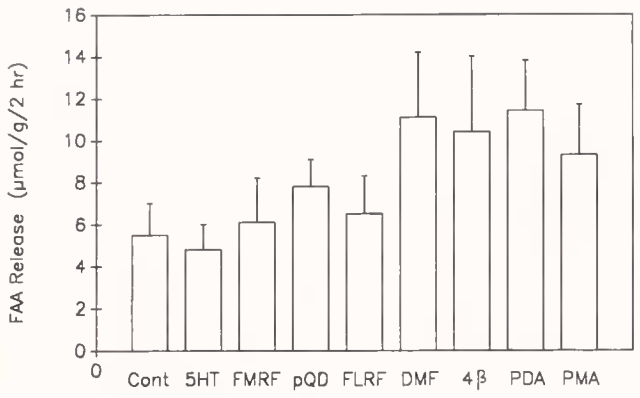

Figure 6. The effects of various agents on the release of amino acids from *Mercenaria* ventricles transferred to isosmotic seawater (1000 mOsm). Each bar represents the mean of five ventricles; error bars are 1 SD. Treatments: Cont = controls; 5HT = 5-hydroxytryptamine (10^{-6} *M*); FMRF = FMRFamide (10^{-6} *M*); pQD = pQDFLRFamide (10^{-6} *M*); FLRF = FLRFamide (10^{-6} *M*); DMF = dimethylformamide (1.4 m*M*); 4β = 4-beta-phorbol (10^{-7} *M*); PDA = phorbol 12,13-diacetate (10^{-7} *M*); PMA = phorbol 12-acetate,13-myristate (10^{-7} *M*).

The wet weights of ventricles incubated in 500 mOsm SW containing 10^{-6} *M* 5HT are lower than control tissues at 10, 30, and 60 min ($t = 9.72$; $P < 0.001$; $t = 10.7$, $P < 0.001$; $t = 3.17$, $P < 0.01$; respectively) (Figs. 7a, b). Ventricles incubated in 500 mOsm SW containing forskolin (10^{-5} *M*) gain significantly more weight than controls in the first 10 min ($t = 63.2$, $P < 0.001$), but there is no difference in wet weight gain 30 and 60 min after transfer (Figs. 7a, b).

Phorbol 12-acetate,13-myristate (10^{-7} *M*) reduces the changes in weight relative to control tissues at 10, 30, and 60 min ($t = 38.3$, $P < 0.001$; $t = 17.9$, $P < 0.001$; $t = 6.55$, $P < 0.001$, respectively) following transfer from 1000 mOsm SW to 500 mOsm SW (Fig. 7c). Tissues transferred from 1000 mOsm SW to 500 mOsm SW containing phorbol 12,13-diacetate show significantly higher ($t = 124.4$, $P < 0.001$) weight gain than control tissues 10 min after transfer, and significantly lower ($t = 7.83$, $P < 0.001$) weight gain 30 min after transfer. There is no significant difference between these tissues and controls 60 min after transfer (Figs. 7a, c).

The changes in wet weight of ventricles transferred from 1000 mOsm SW to isosmotic SW containing FMRFamide, 5HT, and phorbol esters are shown in Figure 8. There is no significant change in wet weight in control tissues following transfer, nor did any of the treatments effect significant differences in weight change relative to control tissues.

Discussion

The release of amino acids from *Mercenaria* cardiomyocytes is potentiated by 5HT and by the molluscan neuropeptide FMRFamide and its naturally occurring analogs. These agents also cause a reduction in the time required for ventricles exposed to hypoosmotic media to

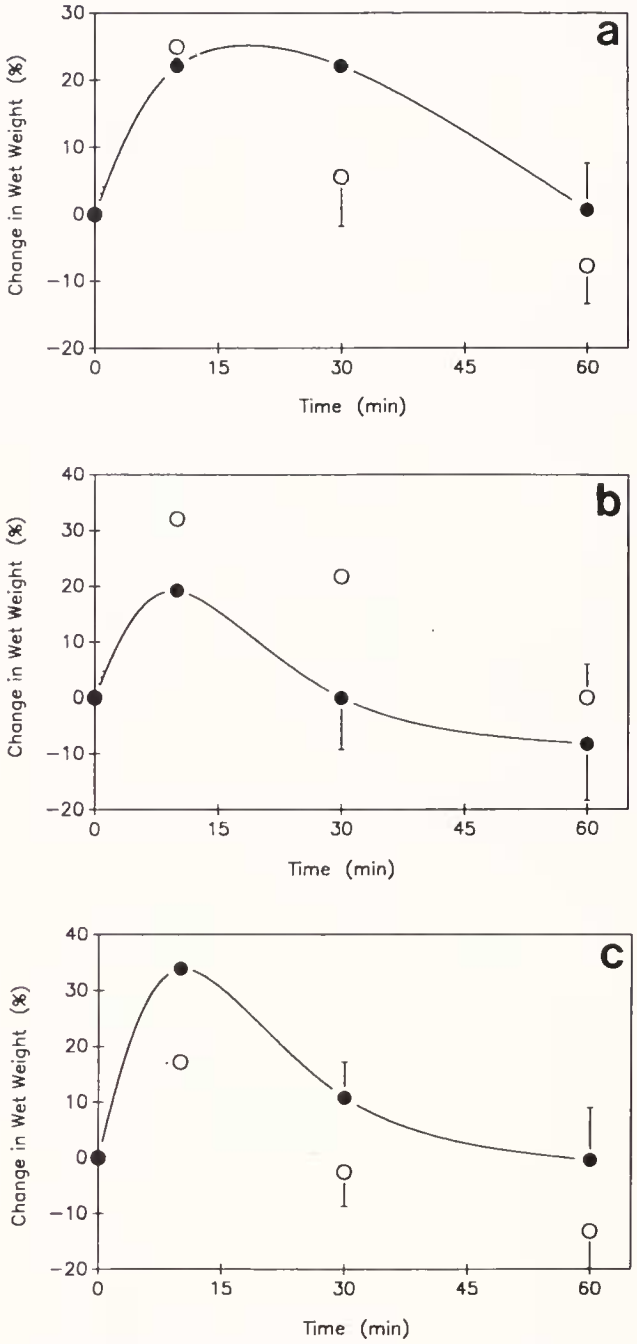

Figure 7. The effects of various agents on the time course of changes in wet weight of *Mercenaria* ventricles transferred from 1000 mOsm seawater to 500 mOsm SW. Each point is the mean of 10 ventricles; error bars are 1 SD. Treatments are indicated as follows: 7a—solid circles = controls, open circles = FMRFamide (10^{-6} *M*); 7b—solid circles = 5-hydroxytryptamine (10^{-6} *M*), open circles = forskolin (10^{-5} *M*); 7c—solid circles = phorbol 12,13-diacetate (10^{-7} *M*), open circles = phorbol 12-acetate,13-myristate (10^{-7} *M*).

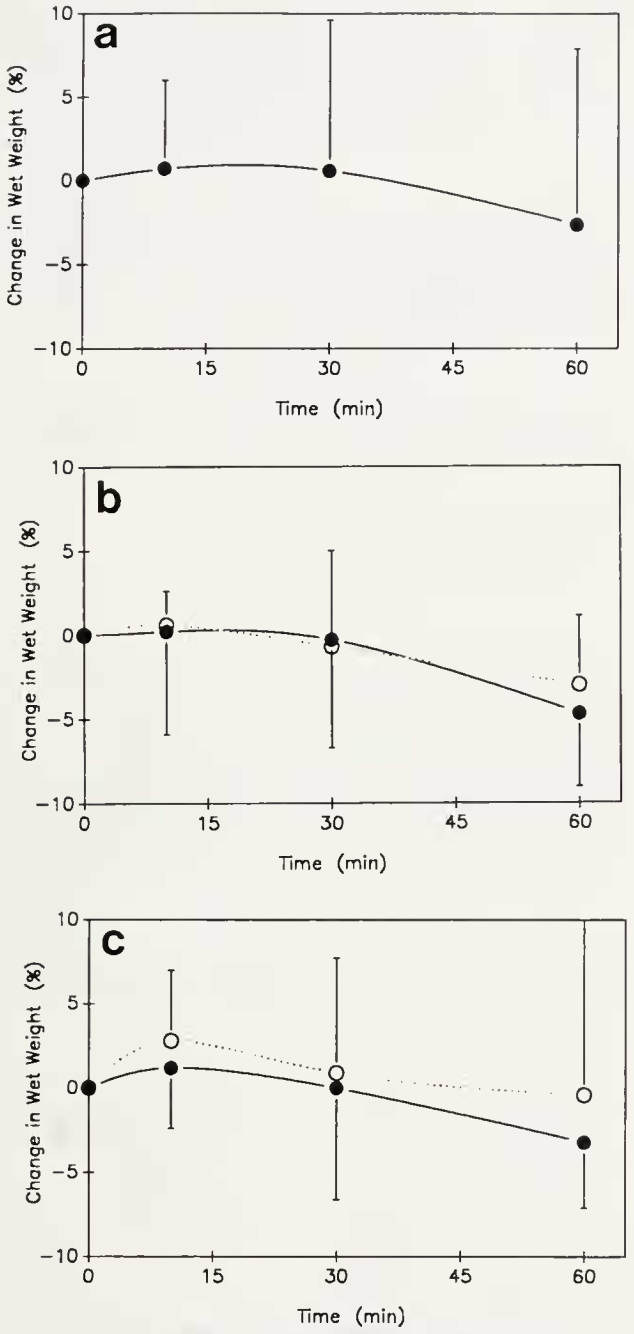

Figure 8. The effects of various agents on the time course of changes in wet weight of *Mercenaria* ventricles transferred to isosmotic seawater (1000 mOsm). Each point is the mean of 10 ventricles; error bars are 1 SD. Treatments are indicated as follows: 8a—solid circles = controls; 8b—solid circles = FMRFamide (10^{-6} *M*), open circles = 5-hydroxytryptamine (10^{-6} *M*); 8c—solid circles = phorbol 12,13-diacetate (10^{-7} *M*), open circles = phorbol 12-acetate,13-myristate (10^{-7} *M*).

volume regulate. The effect of 5HT on amino acid release is mediated by 5HT receptors, since this effect is blocked by UML. The effective concentrations for the potentiation of amino acid release by 5HT and FMRFamide are in the nanomolar range; the concentration of FMRFamide in the hemolymph of the clam *Macrocallista nimbosa* is also in this range (Nagle, 1982).

Both 5HT and FMRFamide stimulate the mechanical activity of isolated *Mercenaria* ventricles (Price and Greenberg, 1980); the cardioexcitatory effects and the potentiation of the volume regulatory response in hypoosmotic media might be due to similar intracellular mechanisms. However, the release of amino acids from ventricles in isosmotic seawater is not affected by either 5HT or FMRFamide. Thus, the chain of events responsible for the cardioexcitatory effects cannot be identical to that responsible for the increase in the release of amino acids and the decrease in the time necessary for the volume regulatory response of the tissues.

Previous studies suggest that stimulation of the mechanical activity of *Mercenaria* ventricles by 5HT and FMRFamide involves an increased sequestration of Ca^{++} ions by the sarcoplasmic reticulum effected by an increase in the intracellular level of c-AMP (Higgins, 1974; Higgins and Greenberg, 1974; Higgins *et al.*, 1978), but the cardioexcitatory effects of 5HT and FMRFamide cannot be completely explained by this mechanism (Paciotti and Higgins, 1985; Deaton and Gray, 1989). The failure of forskolin to affect the release of acids suggests that c-AMP is not involved in the potentiation of this process by 5HT and FMRFamide.

Phorbol esters stimulate protein kinase C, which is also stimulated by diacylglycerol, one component of the phosphoinositol cellular signal transduction system (Nishizuka, 1984; Berridge, 1986). Phorbol esters potentiate the volume regulatory response and increase the release of amino acids of ventricles exposed to dilute media. The phorbol esters only effect increases in the release of amino acids from tissues exposed to hypoosmotic media. The biologically inert compound, 4-β phorbol, also has no effect on amino acid release. The regulatory volume decrease of red blood cells from the clam *Noetia ponderosa* is potentiated by PMA, which appears to affect cytoplasmic K^+ levels (Pierce *et al.*, 1989). Phorbol esters also potentiate the release of amino acids from elasmobranch erythrocytes incubated in hypoosmotic media (Leite and Goldstein, 1987), and mimic the effect of osmotic shrinking on the ion exchangers responsible for adjustment to hyperosmotic stress by cultured lymphocytes (Grinstein *et al.*, 1986). There is, however, no increase in the IP_3 levels in skate red blood cells exposed to hypoosmotic medium (McConnell and Goldstein, 1988). These results suggest that protein kinase C is involved in volume regulation; but the role, if any, of IP_3, is not clear.

Incubation of ventricles from the mussel *Geukensia demissa* in isosmotic medium containing 0.54 m*M* KCl,

which depolarizes the cells by about 60 mV (Wilkens, 1972), increases the release of amino acids from 15 to 21 μmol/g dry wt (Pierce and Greenberg, 1976). These observations raise the possibility that the effects of 5-HT, FMRFamide, and phorbol esters on the release of amino acids might be due simply to depolarization of the cells. This seems unlikely for two reasons. First, there was no increase in the amino acid release from ventricles treated with 5-HT, FMRFamide, or phorbol esters in isosmotic seawater. Second, large doses (10^{-6} *M*) of 5-HT have little effect on the membrane potential of either *G. demissa* or *Mytilus edulis* cardiac muscle cells (Irisawa *et al.*, 1973). However, the effects of FMRFamide and 5-HT on the membrane potential of *Mercenaria* cardiomyocytes are unknown.

In summary, the regulatory volume decrease (RVD) of *Mercenaria* ventricular cells exposed to hypoosmotic media may be mediated by the activation of protein kinase C. The potentiation of the RVD and loss of free amino acids from isolated ventricles by 5-HT and FMRFamide raises the possibility that the response of bivalve tissues to hypoosmotic stress may be modulated by neuronal or neurohormonal control.

Acknowledgments

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